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INDUSTRIAL WASTEWATER AND GLYCEROL AS FEEDSTOCK FOR SUSTAINABLE PHA PRODUCTION BY *BACILLUS* AND *ACINETOBACTER* SP.

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ABSTRACT

Polyhydroxyalkanoates (PHA) are eco-friendly biopolymers used as an alternative to petroleum-based plastics. The present study focused on screening PHA-producing bacteria and optimizing polymer production using glycerol and wastewater as feedstocks. Three PHA-producing bacterial strains (SS8, SS10, and SW9) were screened and identified to be *Acinetobacter venetianus* SS8 (MF285609), *Bacillus pumilus* SW9 (MF285610), and *Bacillus pumilus* SS10 (MF285611**).** Growth kinetics of PHA producers were observed at different time intervals with varying carbon sources like 2% glucose, 2% glycerol, 0.2% glycerol, and wastewater. The highest amount of PHA was extracted from strain SS10, i.e., 69.2% in PDA with 0.2% glycerol. The bacterial strain SS8 produced 36% PHA after 24 hours of growth in IWW-supplemented media. FTIR analysis of the extracted PHA showed a carbonyl (C=O) ester bond at 1721, 1722, and 1722 cm^{-1} confirming that the bacteria produce scl PHA. The presence of the *phaR* gene indicates that the PHB operon is involved in the production of scl-PHA. The study signifies the extraction of biodegradable polymers that decrease the burden on petroleum oil and helps create a cleaner environment.

Keywords: Bioplastics, *Bacillus pumilus*, *Acinetobacter venetianus*, Growth characters, Polyhydroxyalkanoate, Tannery wastewater.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are naturally occurring biopolymers bacteria produce as a carbon and energy storage compound (Moradali and Rehm, 2020). These biopolymers are biodegradable and have properties similar to synthetic plastics, making them a potential alternative to petroleum-based plastics (Behera *et al*., 2022). There are 155 types of PHA monomers, each subunit with a different monomer repeats number and side groups (Ferreira and Åkesson, 2020). Various microorganisms produce PHA (e.g., *Ralstonia eutropha* (Bhatia *et al*., 2019) *Bacillus* (Lee *et al*., 2022; Patil *et al*., 2022; Pinyaphong and Sriburi, 2022), *Pseudomonas* (Mahato *et al*., 2021), *Acinetobacter* (Idris *et al*., 2022), and *Rhodococcus* sp. (Trakunjae *et al*., 2021)), about 90% of the cell dry weight (Bong *et al*., 2021).

Gram-positive bacteria are favored to produce PHA because of their potential to produce large amounts of peptidoglycan, which can aid as a copolymer. However, Gram-negative bacteria have thinner cell walls, and it is easier to extract the PHAs, producing higher PHA yields (Valappil *et al*., 2007). In either case, the Gram-positive and Gram-negative strains were chosen to utilize specific sugars and can produce high-yield polyhydroxyalkanoates (PHAs). The significance of this biodegradable polymer will decrease the burden on petroleum oil and help create a cleaner environment (Khatami *et al*., 2021).

Researchers face the challenge of getting a high yield of PHA, making it difficult to commercialize PHA on a larger scale (Blanco *et al*., 2021). Many reasons lead to the low yield of PHA, and one of the major ones is using expensive carbon sources. Researchers have tried using waste materials as carbon sources for sustainable PHA production (Szacherska *et al*., 2021; Yadav *et al*., 2021; Khan *et al*., 2022; Vicente *et al*., 2023). This study used industrial wastewater (IWW) and waste glycerol as carbon sources to optimize PHA production by *Bacillus pumilus* and *Acinetobacter venetianus.* Bacteria use glycerol as a carbon source for PHA production by converting it to acetyl-CoA, a key precursor molecule in the PHA synthesis of PHAs. The conversion of glycerol to acetyl-CoA is a multi-step process that involves several enzymes and metabolic pathways. Glycerol is converted to glycerol-3-phosphate, which is then oxidized to dihydroxyacetone phosphate by the enzyme glycerol-3-phosphate dehydrogenase, releasing a molecule of NADH. Dihydroxyacetone phosphate is then converted to pyruvate, which

is then converted to acetyl-CoA. Once acetyl-CoA is produced, the bacteria use it to synthesize PHAs through a series of enzymatic reactions catalyzed by the enzymes of the PHA synthesis pathway. The specific enzymes and metabolic pathways involved in PHA synthesis can vary depending on the type of bacteria used, and the specific PHA produced (Bryan *et al*., 2022; Koller and Obruča, 2022).

Industrial wastewater can contain volatile fatty acids (VFAs), including acetic acid, propionic acid, butyric acid, and valeric acid (Atasoy and Cetecioglu, 2022). VFAs are produced due to the anaerobic fermentation of organic compounds in the wastewater by certain types of bacteria, such as acidogenic bacteria (Carolina *et al*., 2022). VFAs are useful in PHA production because they can serve as a carbon and energy source for PHA-producing bacteria. Under conditions of nutrient limitation, such as limited nitrogen or phosphorus, bacteria can store excess carbon from VFAs as PHAs (Tu *et al*., 2020). Using VFAs as a carbon source for PHA production, wastewater can be treated and recycled into a valuable resource while producing a biodegradable and sustainable biopolymer (Szacherska *et al*., 2021; Guleria *et al*., 2022). In addition to providing a carbon source for PHA production, VFAs can also help adjust the growth medium's pH, which can be necessary for optimal bacterial growth and PHA production (Vu *et al*., 2022). However, the concentration and composition of VFAs in industrial wastewater can vary widely depending on the source and type of industrial process, which can impact the efficiency and yield of PHA production (Gabriela *et al*., 2021). Therefore, careful selection of bacterial strains and optimization of growth conditions are necessary to achieve high yields of PHAs from industrial wastewater.

Thus, the main objective of the present study is the isolation and characterization of bacterial strains from industrial wastewater, which can produce high-yield PHAs. The isolation of the phaR gene from selected bacterial strains was also carried out to identify the metabolic pathway involved in PHA synthesis.

MATERIALS AND METHODS

Sample Collection and Isolation of Bacterial Strains

The industrial wastewater (IWW) of the DADA enterprise district of Qasoor (31.1855° N, 74.4435° E) was collected in sterile plastic bottles. Similarly, the soil sample was collected from the agricultural land of the University of the Punjab, Lahore (31.4790° N, 74.2662° E) for bacterial strain isolation. The collected samples were serially diluted and inoculated on nutrient agar plates. Isolated colonies with variable morphology were selected for further screening. The nutrient agar medium was used before selective screening to collect as many bacterial strains as possible. The obtained bacterial strains were then screened for polyhydroxyalkanoates (PHA) production using various phenotypic parameters described below.

Phenotypic Screening of PHA Producers

The isolated bacterial strains were screened for PHA production by inoculating on the PHA detention agar (PDA) (Khan and Jamil, 2021) plates containing 0.5 µg ml^{-1} Nile blue (Sadaat and Jamil, 2020). The petri plates were incubated for 24 hours at 37℃ and then viewed in UV light. The Nile blue dyes the intracellular PHA granules, and the bacterial colonies become fluorescent under ultraviolet light (Cánovas *et al*., 2021). Further, the Nile Blue-positive bacterial cells were observed under the microscope using Sudan Black B dye (Javaid *et al*., 2019).

16s rRNA Identification of PHA-Producing Bacteria

DNA of the isolated bacterial strains was isolated using a Thermo Scientific Gene JET DNA Purification Kit. The extracted DNA was gel purified and sent to Ist Base for 16s rRNA gene sequencing. The sequences were then analyzed using Chromas 2.6.6, and phylogenetic trees were constructed using MEGA 11.

Optimization of PHA Production from Different Carbon Sources

The carbon sources used in this experiment for selected bacterial strains were 2% glucose, 2% glycerol, 0.2% glycerol, and industrial wastewater. The PDA medium was supplemented with these carbon sources, and PHA production was compared. A seed culture (10%) of selected bacterial strains was inoculated in different flasks supplemented with carbon sources, 2% glucose, 2% glycerol, 0.2% glycerol, and wastewater. The bacterial culture was incubated at 37 ^{o}C for 74 hours at 150 rpm. PHA was extracted after 24 hours, and a percentage of production of the biopolymer was noted.

PHA Extraction

After the respective time, the media were centrifuged at 6000 rpm for 10 min, and cells were harvested. After freeze-drying the cell palate, the biomass was determined. Then sodium hypochlorite (6.25%) and chloroform (1:40) were added to the pellet (1g) and incubated at 37 ^{o}C in a shaker (120 rpm) for 1 hour. Aqueous and organic layers

were separated using a separating funnel; the upper layer was discarded while the lower transparent layer evaporated to determine the amount of PHA.

%PHA = Weight of PHA/ Weight of biomass x100

Characterization of the PHA

One milligram of partially purified bioplastic was completely dried. Then it was analyzed in an FTIR (Agilent ATR-FTIR spectrometer), a device getting the spectrum within the vary of four, 400 - 4000 cm^{-1} at a resolution of four cm^{-1}.

Isolation and Analysis of Regulatory Gene *phaR*

The *phaR* was amplified; using PCR, and the genomic sequence was analyzed using NCBI Blast, and Chromas. The phylogenetic relationship of the gene with the other genomes was analyzed using MEGA11.

RESULTS

PHA-Producing Bacterial Strains

A total of 20 strains were selected based on their different colony morphology; 11 were from the soil, and nine were from tannery wastewater. The results indicated that among all the strains, SS8, SS10, and SW9 showed fluorescence and hence were selected as PHA producers, as shown in Fig. 1.

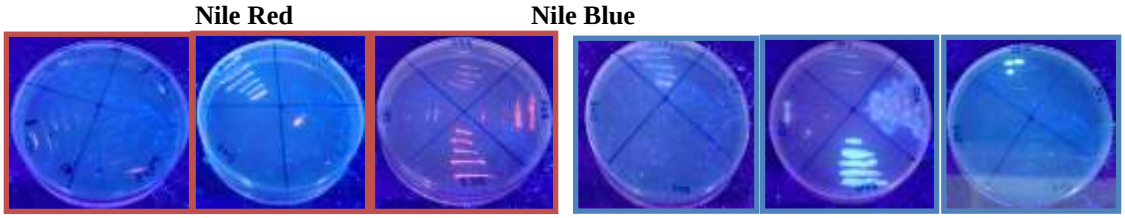

Fig. 1. Screening of PHA strains using Nile red and Nile blue staining.

Sudan black staining was carried out to confirm PHA granules in the bacterial cell. The bacterial strains SS8, SS9, and SS10 were observed to have intracellular PHA granules, as shown in Fig. 2.

Sudan black screening

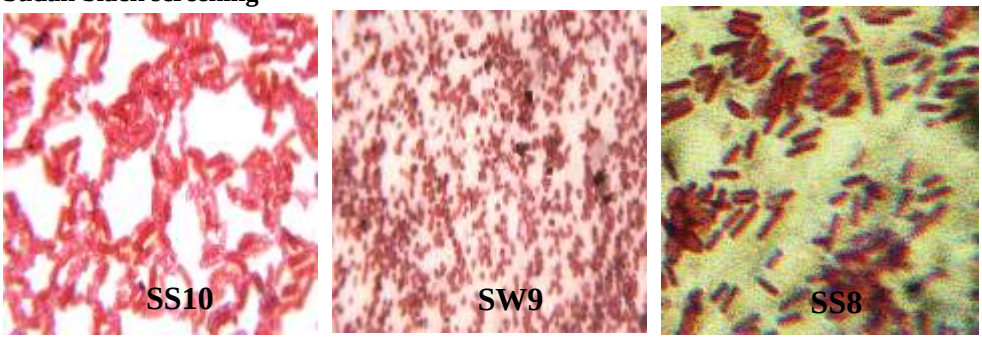

Fig. 2. Sudan black staining of strains showing PHA granules.

Identification of PHA-Producing Bacteria

16S Sequencing

The DNA of the selected strains, i.e., SS8, SS10, and SW9, was isolated. The purified sample was sent to 1stBase for 16s rRNA gene sequencing. NCBI Blast analysis revealed that the sequenced strain SS8 was related to a subspecies of *Acinetobacter venetianus*, whereas strains SS10 and SW9 were related to *Bacillus pumilus*. The GenBank accession numbers of the bacterial strains are; *Acinetobacter venetianus* SS8 (MF285609), *Bacillus pumilus* SW9 (MF285610), and *Bacillus pumilus* SS10 (MF285611).

PCR Amplification of phaR Gene

The *phaR* gene was isolated from the bacterial strain *Acinetobacter venetianus* SS8, and the sequences were analyzed using NCBI BLAST and MEGA11. The phylogenetic analysis revealed that the *phaR* belongs to a pha operon containing *phaC*, *phaB*, and other regulatory genes such as *phaP*, *phaQ*, and *phaJ* (Fig.3). This operon is involved in the biosynthesis of small chain length PHA known as Poly(3-Hydroxybutyrate).

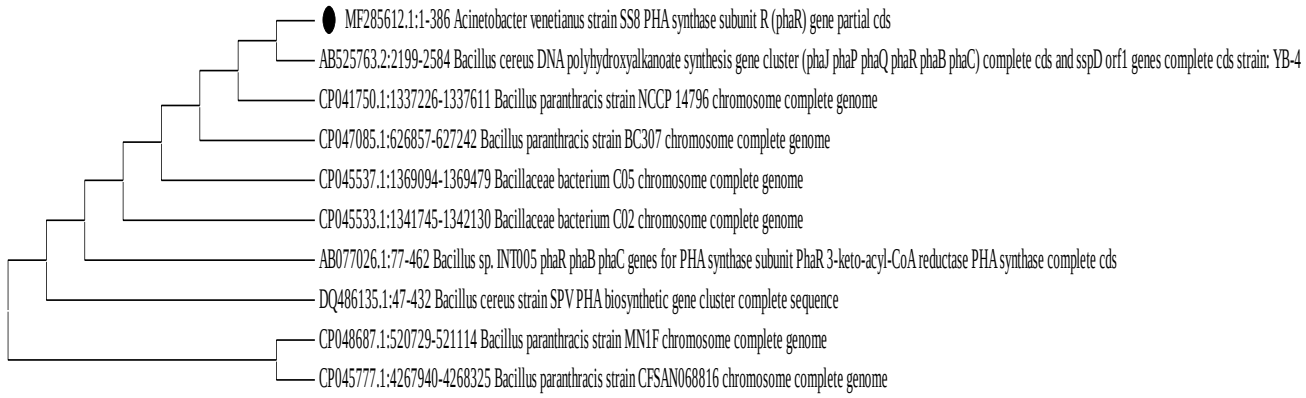

Fig. 3. Phylogenetic analysis of the phaR gene isolated from bacterial strain SS8.

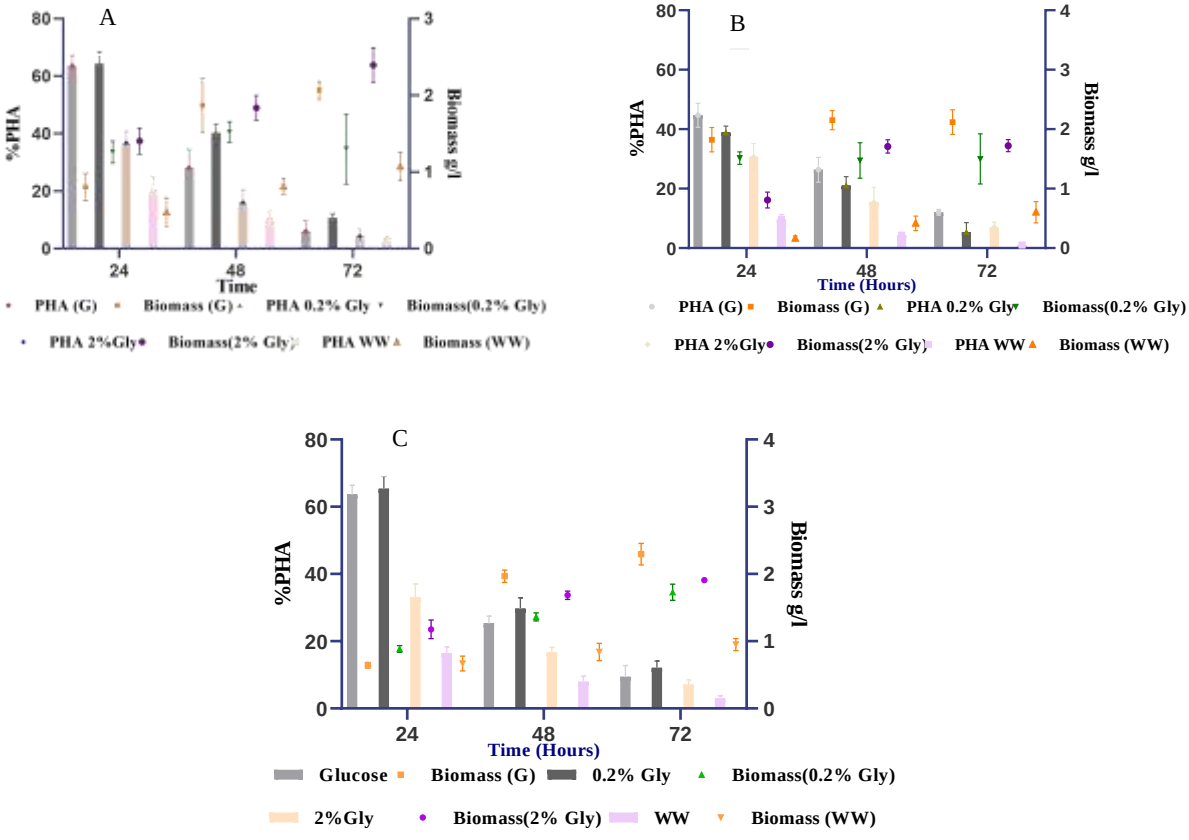

Fig. 4. PHA production on PDA broth with glucose, PDA with 2% glycerol, PDA with 0.2% glycerol, and wastewater with 2% glucose (A) SS8, (B) SS9, (C) SS10.

PHA Production and Optimization

A comparison of PHA production using 2% glucose, 0.2%, 2% glycerol, and 2% IWW was observed. The bacterial strain SS8 produced 63% PHA in the first 24 hours of incubation in glucose-supplemented media. Although the biomass was low (0.8 g/l), the PHA percentage was high. The PHA accumulation gradually decreases to 24 and 6% after 49 and 72 hours of growth. An increase in biomass was observed over time; it was noted that as PHA% decreased, the biomass increased in glucose-supplemented media. The strain SS8 produced 64% PHA when supplemented with 0.2% glycerol after 24 hours of incubation, production higher than 2% glucose. The accumulated PHA was used by the bacterial cells, as the percentage of PHA decreased to 40% and then 10% after 48 and 72 hours of incubation. The PHA production by the strain SS8 was relatively low when supplemented with 2% glycerol and 2% IWW. The bacterial strain SS8 produced 36% and 19% PHA in the first 24 hours of incubation when supplemented with 2% glycerol and IWW independently, as shown in Fig. 4.

Data revealed that SS10 was the highest PHA-producing strain among these three strains, as SS10 produced 63.7% PHA in PDA medium with 0.2% glycerol as a carbon source and 65.2% in 2% glucose. At the same time, SS8 produced the maximum percentage of PHA, i.e., 33.68% in 2% glycerol, 30% in 0.2% of glycerol, 28% of PHA in 2% glucose, and about 28% of PHA in wastewater medium using a carbon source (Fig. 4). The appearance of the extracted PHA was brittle and slightly crystalline, with features related to the Poly(3-Hydroxybutyrate) (Fig. 5).

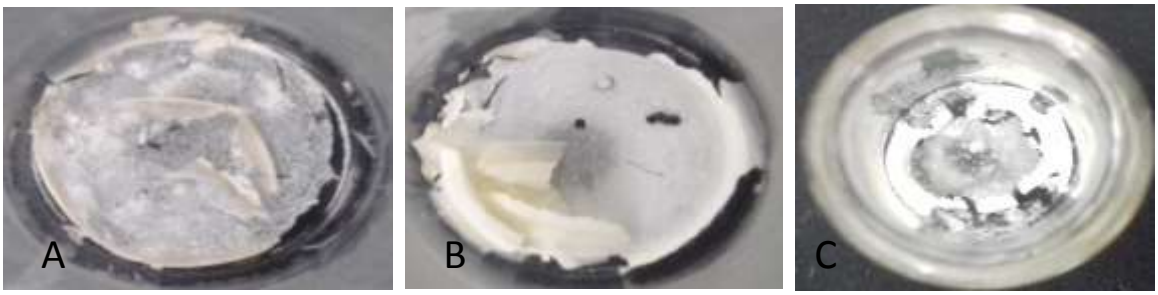

Fig. 5. PHA Polymer produced by strain SS8 (A), SS10 (B), and SW9 (C).

PHA Characterization

FTIR Analysis

Fourier transform infrared (FTIR) spectroscopy analysis of PHA polymer produced by strain SS8, SS10, and SW9 was carried out. The bands in the spectra, regarded as PHA and other molecules such as cellular protein, can be distinguished. The highest peaks were observed at 1721.35, 1722.13, and 1722.17 cm^{-1}. The absorption bands are PHA marker bands assigned to carbonyl (C=O) ester bond stretching vibration. The absorption band at 2955.76cm^{-1} was assigned to the CH2 group (Fig. 6).

DISCUSSION

Natural processes and resources produce bioplastics; microorganisms, especially bacteria, have been manipulated to get biodegradable and biocompatible polymers (Kumar *et al*., 2020). The demand for bioplastics for packaging is rapidly increasing among various industries, particularly the food industry, at a large scale (Muiruri *et al*., 2022). However, the high production cost of PHA limits its industrial applications due to the unavailability of appropriate and economical substrates (Vigneswari *et al*., 2021). Therefore, various studies on PHA production have focused on growth cultures and sustainable substrates (Vicente *et al*., 2023). In the current study, tannery wastewater was used to evaluate the efficiency of the isolated strains to produce PHA.

The bacterial strains accumulating PHA were isolated from tannery wastewater using phenotypic screening methods. The bacterial strains *Acinetobacter venetianus* (SS8), *Bacillus pumilus* (SW9), and *Bacillus pumilus* (SS10) were able to grow favorably on the glycerol and IWW-supplemented media. Previously Munir and Jamil (2015) isolated and identified *"Pseudomonas, Bacillus, Enterobacter, Exiguobacterium,* and *Stenotrophomonas"* using industrial wastewater as a carbon source PHA production.

Using VFAs as a carbon source for PHA production, wastewater can be treated and recycled into a valuable resource while producing a biodegradable and sustainable biopolymer (Battista *et al*., 2020). Bacterial strain SS8 produced maximum PHA (36%) using wastewater as feedstock. Although the IWW provides several substrates for various monomers, the isolated bacterial strains were able to produce only butyric acid.

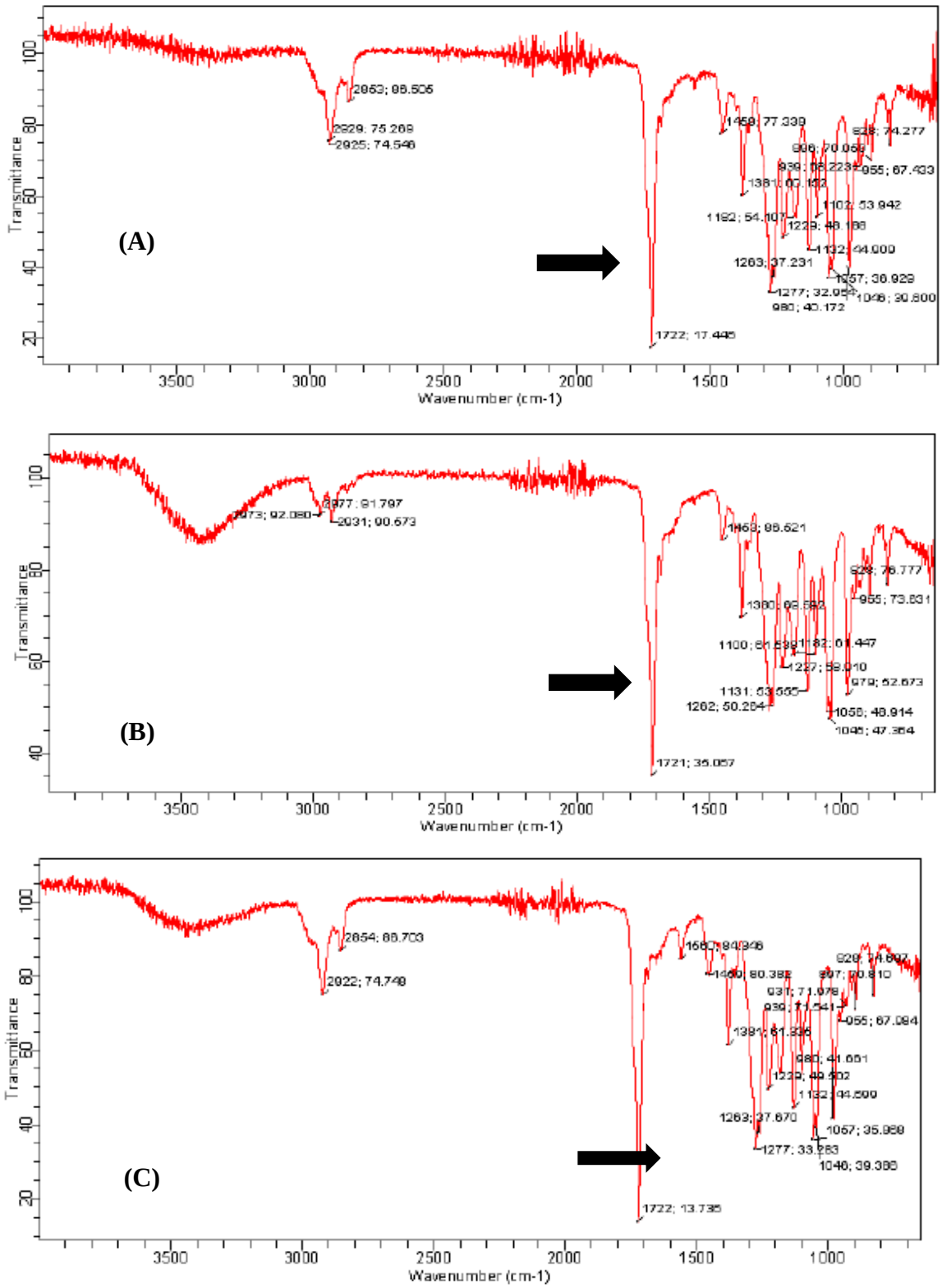

Fig. 6. Fourier transform infrared spectra (FTIR) of the PHA produced by strains SS8 (A), SSS10 (B), and SW9 (C).

The FTIR analysis of the extracted polymer indicated the presence of a carbonyl group at 1720-1722cm^{-1}. The carbonyl group at this range indicated the presence of scl-PHA, more specifically poly(3-Hydroxybutyrate) (Mohammed *et al*., 2020). Similarly, the phaR regulatory gene analysis indicated that the PHA produced by the bacterial strains belongs to Poly(3-Hydroxybutyrate). The phaR gene is a vital component of the regulatory network involved in PHA synthesis. It can play an essential role in controlling the yield and quality of PHAs produced by bacteria. The manipulation of the expression of the phaR gene, either through genetic engineering or environmental control, can be an essential strategy for optimizing PHA production for various applications (Chen *et al*., 2019; Tan *et al*., 2020).

Scl-PHA can substitute traditional petroleum-based plastics in packaging materials, such as bags, films, and containers, providing a sustainable and environment-friendly alternative (Pandey *et al*., 2022; Shlush and Davidovich-Pinhas, 2022). Scl-PHAs are also biocompatible and have low toxicity, making them suitable for medical applications, such as drug delivery systems, sutures, and tissue engineering scaffolds (Elmowafy *et al*., 2019; Pulingam *et al*., 2022). They can also be used in agriculture and horticulture as biodegradable mulch films, plant pots, and other applications (Amelia *et al*., 2019). In the textile industry, Scl-PHAs can substitute synthetic fibers, such as polyester, to produce clothing and other textiles (Muiruri *et al*., 2023). Additionally, Scl-PHAs can be used as a component in adhesives and coatings, providing a sustainable and biodegradable alternative to conventional petroleum-based products (Adeleye *et al*., 2020; Behera *et al*., 2022). Overall, the potential uses of Scl-PHAs are diverse and can provide sustainable alternatives to conventional petroleum-based products in various industries (Tan *et al*., 2021; Acharjee *et al*., 2023). However, further research and development are needed to optimize their properties and production processes for various applications.

CONCLUSIONS

The study signifies using waste materials for sustainable PHA production. Three bacterial strains, SS8, SS9, and SS10, were isolated from industrial wastewater. The bacterial strains were able to produce a significant amount of PHA using glycerol and industrial wastewater. The functional groups present in the polymer indicated it to be scl-PHA. Moreover, the genetic analysis of the phaR gene also revealed similar results. Various environmental wastes should be investigated to determine their suitability for producing PHA and reduce its cost of production. Scl-PHAs have a wide range of potential uses due to their biodegradability, biocompatibility, and thermoplastic properties. Using waste material for PHA production is a wise approach and can lead to sustainable polymer production and control of environmental pollution.

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